

Title

Probiotic Therapy of Neonatal Meningitis and Method of Using *E. coli* Virulence Determinatns

Cross Reference of Related Application

5 This is a Continuation-In-Part application of a non-provisional application, application number 10/123,965, filed April 16, 2002, which is a regular application of a provisional application with application number 60/284,762 filed 04/18/2001.

Statement Regarding Federally Sponsored Research

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Background of the Invention

Field of Invention

 The present invention is related to the fields of infectious disease and molecular biology.

15 Description of Related Arts

Escherichia coli (*E. coli*) is the most common gram negative bacteria causing neonatal meningitis [Huang SH et al., (2000). Microbes and Infection, 2:1137-44; Huang and Jong (2001) Cellular Microbiology 3:277-87]. The pathogenesis of this disease is not completely defined. In the evolutionary process of microbial pathogenesis, gene

acquisition is a major event leading to the emergence and evolution of microbial pathogens. Therefore, microbial pathogens causing infectious diseases usually possess traits or sequence signatures that distinguish them from non-pathogenic or commensal strains. The gut microflora including *E. coli* is acquired rapidly after birth, remains relatively stable throughout the life. These non-pathogenic (probiotic) bacteria are essential for human homeostasis [Huang, S.H. et al.,(2002), *Func Integr Genomics* 1:331-44]. Strains causing meningitis possess traits that distinguish them from commensal strains of *E. coli* and other pathogenic strains such as those causing diarrhea and urinary tract infection (UTI). Characteristically, meningitic strains of *E. coli* are composed of a restricted number of O serogroups (O1, O2, O7, O18, O83), produce S fimbriae, carry the *ibeA* genetic island (GimA) and are predominately carrying K1 capsule (over 84%). The presence of these features implies that meningitic strains possess a defined set of virulence determinants that allow the bacterium to penetrate the blood-brain barrier and get into the central nervous system. Such clusters of potential virulence genes (based on the sequence information), termed genetic islands, have been suggested for meningitic pathogens *N. Meningitidis*, *E. coli* K-1 and *H. influenzae* but have yet to be defined by *in vitro* or *in vivo* experimental approaches (Bloch, C.A. et al., (1996) FEMS Microbiol. Lett.,144: 171-176; Bonacorsi SP et al., (2000) Infect Immun. 68:2096-2101; Chang CC et al.,(2000) Infect Immun 68: 2630-2637; Klee SR et al., (2000) Infect Immun. 68:2082-2095; Rode CK, et al., (1999) Infect Immun. 67:230-236)). The genetic islands such as GimA carry virulence factors that may make *E. coli* shift from the non-pathogenic to meningitic.

Despite advances in antimicrobial chemotherapy, the mortality and morbidity associated with neonatal gram negative bacillary meningitis has remained high. A better understanding of the bacterial genes and proteins that contribute to the pathogenesis of bacterial meningitis (e.g., breaching of the blood brain barrier (BBB) by bacterial pathogens, induction of apoptosis in human endothelial and neural cells) will facilitate the development of novel treatments and prognostic and diagnostic tools for the disease.

Summary of the Present Invention

This invention relates in general to genes and proteins which modulate the virulence of *E. coli* bacteria, in particular meningitic strains of *E. coli*. More specifically, the invention relates to the characterization of the genes in the *ibeA* gene cluster (GimA) and the proteins encoded by these genes, in particular the *ibeA* gene, to compositions comprising the same and applications utilizing the compositions.

It is an object of this invention to provide isolated or purified nucleic acid sequences encoding the genes of the *ibeA* gene cluster.

It is another object of this invention to provide amino acids for the proteins encoded by the genes of the *ibeA* gene cluster.

It is another object of this invention to provide a recombinant molecule comprising a vector and all or part of one or more of the nucleic acid sequences of the *ibeA* gene cluster.

It is another object of this invention to produce recombinant proteins encoded by the *ibeA* gene cluster.

It is another object of this invention to provide methods of diagnosing *E. coli* meningitis.

It is another object of this invention to enhance our understanding of probiotics and their use in the management of infectious diseases, with emphasis on neonatal bacterial meningitis.

It is also an object of this invention to provide vaccines and antimicrobial agents that specifically attack meningitic bacteria but protect probiotic microbes.

These and other objectives, features, and advantages of the present invention will become apparent from the following detailed description, the accompanying drawings, and the appended claims.

Brief Description of the Drawings

5 Figure 1 shows the complete nucleotide sequence and deduced amino acid sequence of the gene *ibeA* from *Escherichia coli* K1 strain. The calculated molecular weight of the full-length protein is 50 kDa. Bolded nucleotide sequences indicate potential ribosome-binding site (RBS), and -10 and -35 promoter regions. The first 15 N-terminal amino acid residues (italicized) completely match the sequence derived from
10 the N-terminal sequencing of the isolated 50-KDa protein expressed in *E. coli* BL21 (DE3) carrying the *ibeA* gene (lane 2 in Fig.2B). Three putative transmembrane domains are underlined. Bolded amino acid sequences denote the partial ORF of *ibeA* reported previously [Huang SH, et al, Infect Immun (1995); 63:4470-5]. Arrows indicate the TnpHoA insertion site (vertical arrows between nt 506 and nt 507) and deletion of ZD1
15 (right arrow, beginning nt151;left arrow beginning at nt 1455) in *ibeA*, respectively.

 Figure 2 shows *In vitro* and *in vivo* biosynthesis of the IbeA protein. *Panel A*, the products of two *in vitro* coupled transcriptions/translations in *Escherichia coli* T7 S30 extract assay system were run in parallel on the same SDS-PAGE (10% polyacrylamide) gel. The sizes of molecular weight markers were indicated to the right. The following
20 templates were added to the reaction mixtures: lane 1, pFN23A carrying a 2.3-kb *ibeA* locus; lane 2, pFN476 (vector). A 50-Kda protein was produced in pFN23A (lane 1) but not in pFN476 (lane 2). *Panel B*, SDS-PAGE (10% polyacrylamide) of total protein extracts of IPTG-induced *Escherichia coli* BL21(DE3) transformed with the vector pFN476 (lane 1), the pFN476-derived plasmid pFN23A bearing the complete ORF of
25 *ibeA* gene (lane 2). A mixture of protein standards (low molecular markers) was run in lane 3, and their molecular mass values (in kDa) are indicated. Additional bands in lane 1

(Fig.2A) and lane 2 (Fig.2B) may result from truncated proteins due to over-expression of IbeA protein.

Figure 3 shows expression and purification of IbeA proteins. *Panel A*, SDS-PAGE (10%polyacrylamide) of total protein extracts of IPTG-induced *Escherichia coli* BL21(DE3)transformed with the vector pET28a(+) (lane 1), the soluble (lane 2) and insoluble proteins (lane3) from the pET28a(+)-derived plasmid pET17A bearing the complete ORF of *ibeA* gene (1.7kb). A mixture of protein standards (low molecular markers) was run in lane 4, and their molecular mass values (in kDa) are indicated. The different patterns of protein bands in the soluble (lane 2) and insoluble (lane 3) fractions showed that a 53-kDa protein is predominately present in the inclusion body (lane 3). *Panel B*, a 53-kDa recombinant IbeA protein with a N-terminal His-tag was expressed in BL21(DE3) carrying pET17A after induction with IPTG. The protein was purified by Ni-NTA Sepharose affinity columns and then refolded by sequential dialysis as described in Materials and Methods. The proteins were resolved on a SDS-polyacrylamide gel, followed by staining with Coomassie Brilliant Blue. Lane 1: purified and refolded IbeA; and lane 2: the molecular weight markers.

Figure 4 shows inhibition of *Escherichia coli* K1 invasion of BMEC by affinity purified and refolded IbeA protein. Confluent monolayers of human BMEC were incubated with either BSA (control)($\mu\text{g}/\text{well}$), or IbeA protein ($\mu\text{g}/\text{well}$) for 1 h at 37°C before addition of bacteria. Invasion assays were carried out as described in the Materials and Methods in the Examples. Each value represents the mean of at least four experiments done in triplicate, and the error bars indicate the standard deviations.

Figure 5 shows Complementation of the non-invasive mutants of E44 with pUC23A and pUC1030 containing *ibeA* locus. *Escherichia coli* E44 is a spontaneous rifampin-resistant mutant of RS218 expressing IbeA. 10A-23 and ZD1 are the TnphoA insertion and isogenic deletion mutants of *ibeA* derived from E44, respectively. Invasion assays were carried out as described in the Materials and Methods in the Examples. The

relative invasion of the mutants compared to the parent strain E44 are shown. Complementation of the *TnphoA* mutant 10A-23 (A) and the isogenic *ibeA* deletion mutant ZD1 (B) was presented. Results are means of four separated experiments; bars represent SD.

5 Figure 6 shows a schematic representation of the overlapping DNA clones covering the 20.3 kb *gimA* and DNA sequencing by primer-walking. Three overlapping clones were identified by screening lambdaGEM-12/RS218 genomic DNA library (A10-8 and A10-30) and PCR cloning. The regions containing *ibeA* common to A10-8 and A10-30, and the overlapping regions between L7 and A10-8 are shown. 30T7 (a-f), 30T7
10 (1-3), 8T3 (a-c), 8T3 (1-6), 10A5-(2-11), 10A3-(1-7), and L7SP1 represent the primers used for DNA sequencing.

 Figure 7 shows the relative genetic location and operon structures of the genetic island *gimA*. Open rectangles indicate the ORFs for the *GimA* and the flanking *E. coli* K12 genes. The orientation of transcription is indicated by arrows. The *GimA* consists of
15 4 operons (*GimA*_{1,2,3,4}).

 Figure 8 shows the ORF annotation by sequence comparison. 13 of 15 ORFs encoded by the genetic island *gimA* in *E. coli* K-1 strain RS218 show significant sequence homology to the corresponding paralogues from *E. coli* K-12 strain.

 Figure 9 shows the phylogenetic trees of 12 gene products in *GimA* based on
20 multiple alignments with ClustalW. ECOK1: *E. coli* K1strain; ECOK12a: *E. coli* K12 strain; ECOK12b: *E. coli* K12 strain; ECOK12c: *E. coli* K12 strain; CORGL: BACSU: MYCTU: HAEIN: *Haemophilus influenzae*; SYNY3: AISC: MAVL: MYCL: ALCEU: *Ralstonia eutropha*; CAEEL: *Caenorhabditis elegans*; DROME: *Drosophila melanogaster*; STRCO: *Streptomyces coelicolor*; TRYBB: VIBPA: PSEPU: BACI:
25 PSEU: CITFR: PICPA: *Pichia pastoris*; SCHPO: *Schizosaccharomyces pombe*; PICAN: *Pichia angusta*; PSEA: ECBK12: GARK12: RHOCA: *Rhodobacter capsulatus*; SALTY:

LACSK: LISMO: STAAU: MYCPN: MCYGE: LYCE: XANCP: *Xanthomonas campestris* pv. *campestris*

Figure 10 shows multiple alignment of 4 sequences of Na(+)/H(+) antiporters, as obtained from CLUSTAL W and DIALIGN. Identical and similar residues in more than 5 52% of the sequences are drawn on black and shaded backgrounds, respectively. From top row to bottom row, the sequences are IbgT from *E. coli* K-1 (ECOK1), Na(+)/H+ antiporter from *H. influenzae* (HAEIN)(Q57007), *B. firmus* (BACFI)(P27611) and *B. subtilis* (BACSU)(P54571).

Figures 11A-11D show the nucleotide sequence for the *ibe A* gene cluster. The 10 start sites for each gene in the cluster is provided in column 4 of Table 4. For example the start site for PptE is 1694 on the complimentary strand. The proteins encoded by the *gimA1* and *gimA3* operons are encoded by a nucleotide sequence complimentary to the one shown in Figure 11A-D.

Figure 12 shows the amino acid sequences for Pgdk (GimA1); PptE (GimA1); 15 PmpT (GimA1); PdaK (GimA1); CgrD (GimA2); CgxT (GimA2); CdID (GimA2); Cnit (GimA2); GcxK (GimA2); GcxR (GimA3); Gc1A (GimA3); GhyI (GimA3); IbgR (GimA4); IbgT (GimA4).

Figure 13 shows induction of apoptosis by the IbeA protein (upper right panel) and inhibition of induction of apoptosis by the IbeA protein by the bacterial permeability- 20 increasing protein (BPI) (lower right panel; ratio of IbeA:BPI was 1;1). The upper left panel shows incubation of the endothelial cells with BSA and IbeB proteins. the lower left panel shows incubation of the endothelial cells with BPI alone.

Detailed Description of the Invention

The term “nucleotide sequence” refers to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and also cDNA.

Substantially homologous as used herein refers to substantial correspondence
5 between a nucleic acid gene sequence of the *IbeA* gene cluster (Figure 1; Figure 11). Substantially homologous means about 30-100% homology, preferably by about 60-to 67-100% homology, and most preferably about 80-100% homology between an *IbeA* gene cluster sequence and that of any other nucleic acid sequence. In addition, substantially homologous as used herein also refers to substantial correspondence
10 between an amino acid sequence of the *IBEA* gene cluster (shown in Figures 1 and 11) and that of any other amino acid sequence.

The term “modulation” refers to either an increase or a decrease in the expression of a gene transcript or protein or impairment of the activity of the protein.

The term “specifically hybridizable” is used to indicate a sufficient degree of
15 complementarity or precise pairing such that stable and specific binding occurs between the a nucleic acid sequence and the DNA or RNA target. It is understood in the art that the sequence need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable.

The term “corresponds to” refers to homologous to or substantially equivalent to
20 or functionally equivalent to the designated sequence.

The present invention provides nucleic acid sequences for the genes of the *ibeA* gene cluster (Figure 1; Figure 11) which encode 15 proteins (Figure 2; Figure 12) which modulates the virulence of meningitic *E. Coli* and thus the pathogenicity of a meningitic *E. Coli*, such as in neonatal bacterial meningitis. For example, the *IbeA* gene
25 modulates the ability of the meningitic *E. Coli* to penetrate the BBB and the induction of

apoptosis. The nucleic acid sequences for the *IbeA* gene cluster shown in Figures 1 and 11 (Table 4), represent preferred embodiments of the invention. It is, however, understood by one skilled in the art that due to the degeneracy of the genetic code variations in the gene sequences shown in Figures 1 and 11 will still result in a DNA sequence capable of encoding the *IBEA* protein corresponding to that gene sequence. Such DNA sequences are therefore functionally equivalent to the sequences set forth in Figure 1 and 11 and are intended to be encompassed within the present invention. Further, a person of skill in the art will understand that there are naturally occurring allelic variations in a given species of the nucleic acid sequences shown in Figures 1 and 11, these variations are also intended to be encompassed by the present invention.

This invention further includes proteins or polypeptide or analogs thereof having substantially the same function as any one of the proteins of the *IbeA* operon or gene cluster proteins of this invention. Such proteins or polypeptides include, but are not limited to, a fragment of the protein, or a substitution, addition or deletion mutant of a protein. This invention also encompasses proteins or peptides that are substantially homologous to any one of the proteins produced by the *IbeA* gene cluster. Substantially homologous means about 70-100% homology, preferably about 80-100% homology, and most preferably about 90-100% homology between any one of the proteins of the invention and any another amino acid sequence or protein or peptide.

The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to any one of the amino acid sequences specifically shown herein (Figures 1 and 12) in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of any one of the proteins described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as

lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid or another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Examples of such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those proteins or peptides which contain one or more naturally-occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Proteins or polypeptides of the present invention also include any protein or polypeptide having one or more additions and/or deletions or residues relative to a sequence encoded by a nucleotide sequence of the *IbeA* gene cluster.

This invention also provides a recombinant molecule comprising all or part of one or more of the nucleotide sequences (Figures 1 and 11) and a vector. Expression vectors and method of producing expression vectors are well known in the art. Generally, expression vectors suitable for use in the present invention comprise a least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. It will be understood by one skilled in the art the

correct combination of required or preferred expression control elements will depend on the host system chosen.

It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, N.Y.) or commercially available.

Another aspect of this invention relates to a host organism or cell into which the recombinant expression vector has been introduced. Examples of host cells that may be used include, but are not limited to, eukaryotes, such as animal (e.g., endothelial cells, epithelial cells), plant, insect and yeast cells and prokaryotes, such as E. coli. The means by which the vector carrying the gene may be introduced into the cell includes, but is not limited to, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, N.Y.). In a preferred embodiment, eukaryotic expression vectors that function in eukaryotic cells are used. Examples of such vectors include, but are not limited to, retroviral vectors, vaccinia virus vectors, adenovirus vectors, herpes virus vector, fowl pox virus vector, bacterial expression vectors, plasmids, or the baculovirus transfer vectors. preferred vectors include, but are not limited to pET28a and pCMV-HA. Preferred eukaryotic cell lines include, but are not limited to, endothelial or epithelial cells.

In a further embodiment, the recombinant protein expressed by the host cells can be obtained as a crude lysate or can be purified by standard protein purification procedures known in the art which may include differential precipitation, molecular sieve

chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis, affinity, and immunoaffinity chromatography and the like. (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, N.Y). In the case of immunoaffinity chromatography, the recombinant protein may be purified by
5 passage through a column containing a resin which has bound thereto antibodies specific for a protein of the invention (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, N.Y.).

The nucleotide sequences or portions thereof, of this invention are useful as probes for the detection of any one of the genes of the *IbeA* gene cluster or detection of
10 any one of the gene products (e.g., mRNAs) in for example, a biological sample. Isolation of nucleic acids from a biological sample may be performed by standard methodology (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, N.Y.). Detection may be performed by a variety of conventional methodologies standard methodology, including, but not limited to,
15 Northern Blot Analysis, PCR etc (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, N.Y.). The probes of the present invention are preferably labeled to provide for detection. Examples of labels include, but is not limited to, radioactive labels, fluorescent lables, photometric labels or chemical labels (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley
20 and Sons, New York, N.Y.) In a preferred embodiment a biological sample is assayed for the presence of the *IbeA* or *IbeB* genes or gene products. The nucleotide sequences of this invention (Figures 1 and 11) can also be used as probes to isolate homologs in other species.

The nucleotide sequences or portions thereof, of this invention are useful in
25 diagnostic assays for meningitic *E.coli*, in particular neonatal meningitic *E. Coli*. The diagnostic assays may be performed as described above to detect nucleic acid sequences from a biological sample which are complimentary to the nucleic acid sequences of the invention. in a biological sample. By way of example, the diagnostic assay may

comprise an array of the nucleic acids of the invention attached to a support (e.g., dot blots on a nylon hybridization membrane Sambrook et al.,) that is contacted with the nucleic acids isolated from the biological sample nylon. In a preferred embodiment for the diagnostic assay the nucleic acid sequences comprise a microarray. .

5 The nucleic acid sequences of the invention may be utilized as probes in microarrays comprising a solid phase on the surface of which are immobilized a population of the nucleic acids of the invention. Microarrays can be generated in a number of way The probes can be attached to a solid support or surface, which may be made from, for example, glass, plastic (e.g., polypropylene, nylon), polyacrylamide,
10 nitrocellulose, or other materials. Methods for attaching the nucleic acids to the surface of the solid phase include, but are not limited to, printing on glass plates (Schena et al, 1995, Science 270:467-470; DeRisi et al, 1996, Nature Genetics 14:457-460; Shalon et al., 1996, Genome Res. 6:639-645; and Schena et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 93:10539-11286); or ink jet printer; for oligonucleotide synthesis (U.S. application Ser.
15 No. 09/008,120, filed Jan. 16, 1998).

 The microarrays can also be high-density oligonucleotide arrays. Techniques are known for producing arrays containing thousands of oligonucleotides complementary to defined sequences (see, Fodor et al., 1991, Science 251:767-773; Pease et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:5022-5026; Lockhart et al., 1996, Nature Biotechnology
20 14:1675; U.S. Pat. Nos. 5,578,832; 5,556,752; and 5,510,270; Blanchard et al., Biosensors & Bioelectronics 11:687-690). Other methods for making microarrays may also be utilized (Maskos and Southern, 1992, Nuc. Acids. Res. 20:1679-1684; US Patent 6136592; WO 200054883; WO 200055363; WO 200053812; WO 200014273). The microarrays may be used as is or incorporated into a biochip, multiwell or other device.

25 Preferably the microarrays of the present invention comprise, in addition to one or more of the nucleic acids of the present invention, nucleic acids from non-meningitic

strains of *E. coli* as a control. In a preferred embodiment, the entire *IbeA* gene cluster is included in the microarray.

One of skill in the art will understand that the hybridization and wash conditions are chosen so that the nucleic acid sequences to be analyzed by the invention (e.g., the nucleic acids isolated from the biological sample) "specifically bind" or "specifically hybridize" to the nucleic acid sequences the array. Optimal hybridization conditions will depend on the length (e.g., oligomer versus polynucleotide greater than 200 bases) and type (e.g., RNA, or DNA) of probe and target nucleic acids. General parameters for specific (i.e., stringent) hybridization conditions for nucleic acids are described in Sambrook et al., (supra), and in Ausubel et al., 1987, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York).

Examples of biological samples that can be used in the above assays include, but are not limited to, cerebral spinal fluid, blood, urine, biopsy specimens, pathology specimens, and necropsy specimens. The nucleotide sequences of this invention (Figures 1 and 11) can also be used as probes to isolate homologs in other species.

This invention further comprises an antibody or antibodies reactive with the *IBEA* protein or polypeptides or portion thereof. In this embodiment of the invention the antibodies are monoclonal or polyclonal in origin and are produced by conventional methodology (Kohler and Milstein (1975) *Nature* 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam; PCT patent applications: publication number WO 901443, WO 901443 and WO 9014424 and in Huse et al. (1989) *Science* 246:1275-1281). The protein or portions thereof used to generate the antibodies may be isolated from the meningenitic *E. coli* strain K1, recombinantly produced, or commercially synthesized (Merrifield; R. B. (1963) *J. Amer. Soc.* 85:2149). If the portion of the protein selected for generating antibodies is too short to

be antigenic it may be conjugated to a carrier molecule to enhance the antigenicity of the peptide. Examples of carrier molecules, include, but are not limited to, human albumin, bovine albumin and keyhole limpet hemo-cyanin ("Basic and Clinical Immunology" (1991) Stites, D. P. and Terr A. I. (eds) Appleton and Lange, Norwalk Conn., San Mateo, Calif.).

The antibodies of this invention may be used in immunoassays to detect the novel proteins in biological samples. Examples of immunoassays that may be used include, but are not limited to, radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, chemiluminescent assay, immunohistochemical assay and the like. (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Press, New York, N.Y.; Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, N.Y.). Biological samples appropriate for such detection assays include, but are not limited to, blood samples, cerebral spinal fluid, urine, biopsy specimens, pathology specimens, and, necropsy specimens. Proteins may be isolated from biological samples by conventional methods described in (Ausubel et al., (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, N.Y.).

The proteins or portions thereof of the invention may be used as a vaccine. The vaccine may be provided prior to any clinical indicia of bacterial meningitis or during an infection to enhance the patient's own immune response to the meningitic pathogens carrying virulence proteins but not probiotic bacteria. The vaccine, which acts as an immunogen may comprise one or more of the proteins of the invention or portions thereof. Vaccination can be conducted by conventional methods. For example, the immunogen can be used in a suitable diluent such as saline or water, or complete or incomplete adjuvants. Further, the immunogen may or may not be bound to a carrier to make the protein immunogenic. Examples of such carrier molecules include but are not limited to bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), tetanus toxoid, and the like. The immunogen also may be coupled with lipoproteins or

administered in liposomal form or with adjuvants. The immunogen can be administered by any route appropriate such as intravenous, intraperitoneal, intramuscular, subcutaneous, and the like. The immunogen may be administered once or at periodic intervals until a significant titer of anti-*GIM A* immune cells or anti-*GIM A* antibody is produced. Alternatively the expression vectors of the invention may be utilized as vaccines. For example, an expression vector comprising all or part of the gene sequence for the GimA genes including *ibeA*, whose proteins have been shown to be immunogenic virulence factors can be used. The GimA genes can be cloned into a plasmid vector such as pVR1020 (Vical, Inc., San Diego, Calif.). The vaccine may be tested in an animal model such as mice. This DNA vaccine can be delivered to mice by intradermal inoculation and the antibody titers in the antisera from the immunized mice measured by enzyme-linked immunosorbent assay. The elicited antibodies can also be tested by immunoblotting with GimA proteins including IbeA. Following the initial immunization and a few (e.g., 2-4) consecutive boosts, each at 2-week intervals, protection can be tested in a neonatal mouse model of *E. coli* meningitis.

The antibodies of the invention can also be administered to a subject as anti-meningitic bacterial agents. Preferably the antibodies administered are designed so to minimize an adverse immune response to the antibody itself (e.g., chimeric antibodies, humanizes antibodies; Robinson et al., International Patent Application 184,187; Taniguchi M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al., 1987 Proc. Natl. Acad. Sci. USA 84:3439; Nishimura et al., 1987 Canc. Res. 47:999; Wood et al., 1985 Nature 314:446; Shaw et al., 1988 J. Natl. Cancer Inst. 80: 15553 ;Morrison S., 1985 Science 229:1202 and by Oi et al., 1986 BioTechniques 4:214.).

While it is possible for the vaccines or antibodies be administered in a pure or substantially pure form, it is preferable to present it as a pharmaceutical composition, formulation or preparation. The formulations of the present invention, are for both

veterinary and human use, comprises one or more of the vaccines or antibodies of the present invention together with one or more pharmaceutically acceptable carriers and, optionally, other active agents (e.g., additional antigens for a multivalent vaccine, antibiotics, BPI etc) or therapeutic ingredients. The carrier(s) must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.. The characteristics of the carrier will depend on the route of administration. Such a composition may additionally contain carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The formulations may be prepared by any method well-known in the pharmaceutical art.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in a effective amount and a variety of dosage forms. For example, an effective concentration of the compositions of the invention may be administered orally, topically, intraocularly, parenterally, intranasally, intravenously, intramuscularly, subcutaneously, transdermally or by any other effective means. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intraperitoneal, oral, intercranial, cerebrospinal fluid, pleural cavity, ocular, or topical (lotion on the skin) administration. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in a effective amount and a variety of dosage forms.

This invention also relates to a method of treating bacterial meningitis, in particular neonatal meningitis with probiotics/prebiotics and the bactericidal/permeability-increasing protein (BPI). Probiotics and prebiotics are known to enhance normal microflora by replenishing suppressed nonpathogenic bacteria and inhibiting the growth of microbial pathogens. For example, *Lactobacillus casei* GG (LGG) has been used in the treatment of a variety of infant and childhood intestinal disorders, including diarrhea, malabsorption, and *Clostridium difficile* colitis [Mattar, A.F. et al., (2002), *Pediatr Surg Int.* 18:586-90.]. It is suggested that in the breast-fed infant, elevated gut *Bifidobacterium* may provide health advantages in comparison with formula-fed infants. Probiotic bacteria

such as *Lactobacillus*, *Bifidobacterium* and *Bacteroides thetaiotaomicron* may suppress the growth of meningitic pathogens. Treatment with probiotics may be tested in a neonatal murine model of *E. coli* meningitis. BPI binds with high affinity to endotoxin and, as demonstrated herein(see Example 13) blocks IbeA-induced apoptosis. Treatment with BPI may first be tested in a neonatal murine model of *E. coli* meningitis. Dosages for human clinical trials may be based on clinical trials in progress utilizing BPI. One of skill in the art will appreciate that individualization of dosage may be required to achieve the maximum response for a given subject. It is further understood by one skilled in the art that the dosage administered to a individual being treated may vary depending on the individual's age, severity or stage of the infection and response to the course of treatment. One skilled in the art will know the clinical parameters to evaluate to determine proper dosage for the subject being treated by the methods described herein. Such dosages may be administered as often as necessary and for the period of time judged necessary by the physician.

This invention also relates to a screening assay for assessing the therapeutic potential of a candidate agent for inhibiting the apoptotic activity of the IBEA protein. By way of example, the therapeutic potential of a candidate agent may be assessed by the assay described in Example 13. A variety of cells may be used in this assay, including, but not limited to, human cells (e.g., endothelial or epithelial). The candidate agents suitable for assaying in the methods of the subject application may be any type of molecule from, for example, chemical, nutritional or biological sources. The candidate agent may be a naturally occurring or synthetically produced. For example, the candidate agent may encompass numerous chemical classes, though typically they are organic molecule, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 Daltons. Such molecules may comprise functional groups necessary for structural interaction with proteins or nucleic acids. By way of example, chemical agents may be novel, untested chemicals, agonists, antagonists, or modifications of known therapeutic agents.

The agents may also be found among biomolecules including, but not limited to, peptides, saccharides, fatty acids, antibodies, steroids, purines, pyrimidines, toxins conjugated cytokines, derivatives or structural analogs thereof or a molecule manufactured to mimic the effect of a biological response modifier. Examples of agents from nutritional sources include, but is not limited to, extracts from plant or animal sources or extracts thereof. Agents also include antisense oligonucleotides, including antisense peptide nucleic acids (Good et al., (2001) *Nature Biotechnology* 19: 360)

The agents may be obtained from a wide variety of sources including libraries of synthetic or natural compounds. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are available or readily produced, natural or synthetically produced libraries or compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to random or directed chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Also provided are kits for performing the assays of the invention (e.g., the diagnostic assay). Such kits may comprise the microarrays of the invention. The microarrays may be incorporated into a biochip or multiwell configuration or any other configuration. The kits may further comprise one or more additional reagents for performing the assay such as for example buffers, primers, enzymes, labels and the like. The kits may further comprise, or be packaged with, an instrument for assisting with the performance or analysis of the assay. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle. The kits of the invention may also include an instruction sheet defining administration of the antisense oligonucleotides. The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the

desired vials are retained. Other instrumentation includes devices that permit the reading or monitoring of reactions.

All books, articles, and patents referenced herein are incorporated by reference. The following examples illustrate various aspects of the invention and in no way intended
5 to limit the scope thereof.

EXAMPLES

Example 1-6

In order to identify *Escherichia coli* structures that contribute to the invasion of BMEC, we have previously used transposon TnphoA mutagenesis to generate a
10 collection of noninvasive mutants. Four noninvasive mutants, 10A-23, 7A-33, 23A-20, and 27A-6 with a single TnphoA insertion in *ibeA*, *ibeB*, *yijP*, and *aslA*, respectively, were found to be significantly less invasive in BMEC monolayer *in vitro* and in the newborn rat model of hematogenous *Escherichia coli* meningitis (Hoffman JA, et al, Infect Immun (2000) 68:5062-7; Huang SH, et al, Infect Immun (1995); 63:4470-5;
15 Huang SH, et al, Infect Immun (1999); 67:2103-9; Wang Y, et al, Infect Immun (1999); 67:4751-6).

The partial internal sequence of *ibeA* (*ibe10*) gene encoding an 8.2-kDa protein region has been cloned by PCR [Huang SH, et al, Infect Immun (1995); 63:4470-5] and its recombinant protein was able to inhibit *Escherichia coli* K1 invasion of BMEC
20 monolayers. The *ibeA* gene has been found commonly in cerebrospinal fluid (CSF) isolates of *Escherichia coli* K1 (e.g., C5 and RS218), whereas laboratory strains of *Escherichia coli* K12 (e.g., DH5 α and HB101) as well as noninvasive *Escherichia coli* K1 (e.g., E412) lack *ibeA* [Huang SH, et al, Infect Immun (1995); 63:4470-5; Bingen E, et al, J Infect Dis (1998); 177:642-50; Bonacorsi SP, et al, Infect Immun (2000);

68:2096-101]. In addition, we have identified an approximately 55-kDa receptor protein (IbeAR) on the surface of BMEC that interacts with *Escherichia coli* invasion protein IbeA by using IbeA-Ni-Sepharose affinity chromatography [Prasadarao NV, et al, Infect Immun (1999); 67:1131-8], suggesting that IbeA contributes to *Escherichia coli* K1 invasion of BMEC via a ligand-receptor interaction.

To assess the importance of IbeA-mediated invasion in the crossing of the blood-brain barrier, the biological activity of the invasion protein encoded by the full-length gene and the isogenic deletion mutant of *ibeA* should be tested. In this work, we further characterized *ibeA* gene and its invasion protein by chromosomal gene replacement, complementation, *in vitro* translation, and *in vivo* protein expression.

Materials and Methods for Example 1-6

Bacterial strains, plasmids and media. Bacterial strain, plasmid vectors, and their relevant characteristics are described in Table 1. Mutant strains used in this study were derived from E44,a spontaneous rifampin-resistant mutant of a CSF isolate of K1 encapsulated *Escherichia coli* RS218 (O18:K1:H7), which has been characterized [Kim, KS, et al, J Clin Invest (1992); 90:897-905. Huang SH, et al, Infect Immun (1995); 63:4470-5. Silver RP, et al, Infect Immun (1980); 29:200-6]. DH5 α was used as the host strain in subcloning and preparation of plasmids for DNA sequence determination. BL21(DE3) carrying T7 RNA polymerase gene was the host strain for protein expression of IbeA. SM10(λ pir) and DH5 α (λ pir) were utilized for making isogenic deletion mutants of *ibeA* [Donnenberg MS, et al, Infect Immun (1990); 58:1565-71. Donnenberg MS, et al, Infect Immun. (1991); 59:4310-7]. Strains containing plasmids were grown at 37⁰C in L broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) with ampicillin (100 μ g/ml), kanamycin (50 μ g/ml) and rifampin (100 μ g/ml) for positive selection of

plasmids or bacterial strains (see Table 1). Bacteria were cultured in L broth and stored in L broth plus 20% glycerol at -70°C.

Chemicals and enzymes: Restriction endonucleases, T4 DNA ligase, and other enzymes were purchased from New England Biolabs (Beverly, MA) unless otherwise noted. Chemicals were purchased from Sigma (St. Louis, MO). All isotopes were obtained from New England Nuclear Corp. (Boston, MA). Reagents for preparation of DNA sequencing gels were ultra pure quality from National Diagnostics (Atlanta, GA). The reagents for DNA sequencing reaction with Sequenase and other chemicals were purchased from United States Biochemical Corp. (Cleveland, OH). DNA sequencing kits with dye terminators were obtained from PE Applied Biosystem (Foster City, CA).

Extraction and manipulation of plasmids and subcloning. All genetic manipulations were performed by standard methods [Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York; Cold Spring Harbor Laboratory Press, 1989:1.1-18.57]. Plasmid DNA was extracted by using a Plasmid Mini kit (Qiagen Inc., Chatsworth, CA). Purification of DNA fragments and extraction from agarose gel slices were performed with GeneClean (Bio 101, La Jolla, CA). Competent cells of *Escherichia coli* were made in 10% glycerol and transformed with electroporation as previously described [Huang SH, et al, Infect Immun (1995); 63:4470-5; Huang SH, et al, Infect Immun (1999); 67:2103-9].

Invasion assays: Invasion assays were performed in human BMECs as previously described [Huang SH, et al, Infect Immun (1995); 63:4470-5. Huang SH, et al, Infect Immun (1999); 67:2103-9. Kim KS, et al, Subcell Biochem (2000); 33:47-59. Stins MF, et al, Am J Pathol. (1994); 145:1228-36. Stins MF, et al, J Neuroimmunol (1997); 76:81-90]. Approximately 10^7 bacteria were added to confluent monolayers of BMECs with a multiplicity of infection of 100. A percent invasion was calculated by $[100 \times (\text{number of intracellular bacteria recovered})/(\text{number of bacteria inoculated})]$. The results were

expressed as relative invasion (percent invasion as compared to the invasion of the parent *Escherichia coli* K1 strain).

Construction and screening of a genomic library of Escherichia coli RS218. High molecular weight chromosomal DNA was purified from *Escherichia coli* K1 strain RS218 as previously described [Huang SH, et al, Infect Immun (1995); 63:4470-5]. Genomic DNA was partially digested with *Sau3AI* (New England Biolabs, Beverly, MA) (15 to 23 kb) and then partially filled-in with dGTP and dATP. This DNA with a 5' protruding overhang (5'-GA-3') was ligated into LambdaGEMTM-12 arms with a compatible *XhoI* Half-site (5'-TC-3'). Ligation and packaging of recombinant lambda phage were performed according to the manufacturers instructions (Promega, Madison, WI). The *Escherichia coli* genomic library was screened by DNA hybridization [Huang SH, et al, Infect Immun (1995); 63:4470-5] to identify phage clones that contained *ibeA*. A 0.58-kb *ibeA* DNA fragment in pCIB10B [Huang SH, et al, Infect Immun (1995); 63:4470-5] was released with *EcoRI* and purified by preparative agarose electrophoresis and GeneClean (Bio101, La Jolla, CA), labeled with [α^{32} P]dCTP by using an oligolabeling kit (Pharmacia, Peapack, NJ), and used as a probe in the screening ($>1 \times 10^8$ cpm/ μ g). The phage plaques were replicated onto Nylon filters, UV-linked and hybridized as described previously [Huang SH, et al, Infect Immun (1995); 63:4470-5. Huang SH, et al, DNA Cell Biol (1994); 13:461-71]. Plaques hybridizing to the probe were identified by autoradiography and then purified.

In Vitro Transcription and Translation. In order to determine the size of *ibeA* ORF, the DNA fragments containing *ibeA* gene were transcribed and translated *in vitro* using an *Escherichia coli* T7 S30 extract assay system according to the manufacturer's instructions (Promega, Madison, WI). The reactions were carried out in 50 μ l of reaction mix supplemented with 20 μ Ci of [35 S]methionine and 2 μ g of purified pFN476 or recombinant pFN23A at 30°C for up to 2 hr . 5 μ l of each reaction containing 35 S-labeled proteins was resolved by 10% SDS-PAGE, and gels were dried and then exposed to Kodak X-Omat film overnight.

Protein expression in vivo and N-terminal sequencing. A 2.3 kb *SphI* DNA fragment carrying *ibeA* was cloned and expressed in a low-copy-number plasmid vector, pFN476, which has been successfully used to perform expression analysis of a dozen cloned chromosomal genes of *Escherichia coli* K12 for producing a definitive expression map [21]. The recombinant plasmid pFN23A and its vector (pFN476) were used to transform an *Escherichia coli* strain, BL21(DE3) which includes an integrated T7 RNA polymerase gene. *Escherichia coli* genes in this vector can be preferentially expressed in BL21 (DE3) by utilizing its T7 promoter [Studier FW, et al, Methods Enzymol (1990); 185:60-89]. In order to eliminate gene expression from chromosome, rifampin was used to shut down *Escherichia coli* polymerase before addition of IPTG. Total proteins were subjected to SDS-10% polyacrylamide gel electrophoresis. After separation, the proteins were transferred onto a PVDF membrane. The band corresponding to a 50 kDa protein over-expressed in the transformants with pFN23A was excised and subjected to N-terminal amino acid sequencing.

DNA sequencing and analysis: The complete nucleotide sequence of *ibeA* was determined by the dideoxy chain termination method of Sanger et al [Sanger F, et al, Proc Natl Acad Sci USA (1977); 74:5463-7] with Sequenase version 2.0 kit from U.S. Biochemicals Corp. (Cleveland, OH) and [³⁵S]dATP(1,000 to 1,500 Ci/mmol) obtained from Du Pont NEN Research Products (Boston, MA). The flanking DNA sequence of the partial *ibeA* gene was determined with primer walking. Both strands of the DNA were re-sequenced by the automated approach with fluorescence labeled nucleotides (373A ABI Automated Sequencer) to ensure the accuracy and the sequence data was analyzed with the DNA analysis program developed by the Genetics Computer Group of the University of Wisconsin. DNA and deduced protein sequences were used to search the DNA and protein databases at the National Center for Biotechnology Information (National Library of medicine, Washington, D.C.) by using the BLAST algorithm.

Construction of isogenic in-frame deletion mutant. In order to determine the role of *ibeA* gene in the pathogenesis of *Escherichia coli* meningitis, the *ibeA* in-frame

deletion mutant was generated by integration of the recombinant suicide plasmid pVZD. pVZD was constructed as follow. Two PCR DNA fragments, Z (0.9-kb) and D (1.2-kb), flanking a 1.3 kb region to be deleted were generated by using two pairs of primers (10A5-4a/10ZM6 for Z and 10D5M/10D3 for D) and then ligated to make a 2.1 kb
5 fragment (ZD) carrying an *ibeA* internal deletion (Table 2). The ZD fragment was subcloned into pCVD442 [17] with *Sma*I. The mutants were obtained by mating E44 with SM10λpir carrying pVZD and selected on LB agar containing ampicillin and rifampin. A single such colony was picked and grown to the late logarithmic phase in LB broth without selection. The dilutions were plated on LB plates containing no NaCl and 5%
10 sucrose. Sucrose-resistant colonies were tested for loss of ampicillin-resistance, indicative of the loss of vector sequence. PCR and DNA sequencing were used to confirm the internal deletion in pVZD and the desired chromosomal gene *ibeA* of the mutant with primers 10A5-3Sa and 10B3-4a (Table 2). Amplification was carried out by using the following cycle profile: 35 cycles: 94⁰C for 1 min, 58⁰C for 1 min and 70⁰C for
15 1.5 min [Kolmodin LA, et al, Methods Mol Biol (1997); 67:1-15].

Complementation analysis. A 2.3-kb *Sph*I fragment containing the complete *ibeA* open reading frame was released from pUC1030 containing an 18 Kb DNA fragment with *Sph*I and subcloned into pUC13. The construct was designated pUC23A. Strain E44 and the mutant ZD1 were transformed with the vector pUC13, and the recombinant
20 plasmids pUC23A and pUC1030. E44 was transformed with pUC13. The transformants were tested for their ability to invade BMEC.

Constructs for making a recombinant IbeA protein with a N-terminal His-tag. The following constructs were made as described previously [Huang SH, et al, Infect Immun (1995); 63:4470-5]. The *Bam*HI-*Eco*RI fragment (1.7 kb) from pCR17A, which encodes *ibeA*, was
25 eluted from 1.0% agarose gel after digestion and then ligated to the same restriction sites of pET28a(+) (Novagen). Expression from this construct (pET17A) resulted in a 33-amino acid peptide (including the T7-Tag and 6 × His-Tag) fusion to the N-terminus of IbeA. The size of the fusion protein was 53-kDa. BL21(DE3) carrying T7 RNA polymerase gene was

used as the host strain for pET17A transformation. Transformants were identified by the predicted phenotypes (kanamycin resistance). The protein expression was induced with 0.5 mM IPTG at 37°C.

Purification of IbeA protein. Small-scale expression and purification of the recombinant protein was carried out according to the manufacturer's instructions (Novagen, Madison, WI). The protein preparations isolated from the supernatants and pellets were resolved on 10% polyacrylamide gel (SDS). Insoluble IbeA protein with a histidine tag was purified by binding to Ni-NTA resin in 8 M urea according to the manufacturer's instructions (Novagen, Madison, WI). The eluted proteins containing 8 M urea were refolded as described previously [Huang SH, et al, Infect Immun (1995); 63:4470-5.]. Purity of the final product was assessed by subjecting the indicated amount of protein to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) by the procedure of Laemmli [Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York; Cold Spring Harbor Laboratory Press, 1989:1.1-18.57]. Protein concentration was determined by using the Bio-Rad protein standard assay reagents according to the manufacturer's instruction. The protein was examined for the effect on the invasion of BMEC by the strain E44. Briefly, BMEC was incubated with the purified IbeA protein or bovine serum albumin (negative control) for 1 hr (0.125 to 1.5 µg/well) at room temperature before adding bacteria and invasion assays were carried out as described above.

Example 1

Isolation and subcloning of large DNA fragment carrying the full-length *ibeA* gene. *Escherichia coli* K1 strain RS218 was used as the source of DNA for cloning experiments. This strain has been shown to invade BMEC and induce meningitis in newborn rats [Huang SH, et al, Infect Immun (1995); 63:4470-5]. To clone the invasion determinants from RS218, a genomic library was constructed in lambdaGEM-12. Using the partial sequence of *ibeA* (0.58 kb) as a probe, approximately 5×10^5 recombinant

phages were screened and two phage clones for *ibeA* were identified. The recombinant phage DNAs were purified and digested with *NotI*. The sizes of the inserts were between 16 and 18 kb. An 18-kb insert containing *ibeA* was subcloned into pUC13 (pUC1030) and pWKS30 (pWKS1030).

5 **Example 2**

Sequence Analysis of *ibeA*. In order to further define the full-length *ibeA* gene, we sequenced the 2.3 kb *SphI* fragment carrying the full-length *ibeA* gene. A single ORF was identified in this region. As shown in Fig. 1, a 1,368-nucleotide open reading frame (ORF) assigned to the *ibeA* gene coded for a protein with 456 amino acids and a
10 calculated molecular mass of 50 kDa (Fig. 1). A minor sequence error in the partial gene has been corrected. No significant sequence homology was observed between *ibeA* and other known genes when DNA and protein databases in GenBank were searched. Potential -10 (CTTATA) and -35 (GTTAAT) promoter regions were found at the 5' noncoding region of *ibeA*.

15 **Example 3**

Nucleotide sequence accession number. The complete nucleotide sequence of *ibeA* has been deposited in the GenBank Nucleotide Sequence Data Library under the GenBank accession number AF289032.

Example 4

20 **Determination of the size of IbeA protein in the *in vitro* transcription/translation and *in vivo* protein expression systems.** The size of IbeA protein was verified by *in vitro* protein expression experiments performed in an *Escherichia coli* T7 S30 extract assay system. In this system *in vitro* transcription and

translation of the DNA fragments containing *ibeA* gene were coupled. Initially, pWKS1030 carrying an 18 kb *ibeA* locus and several restriction fragments derived from pWKS1030 were tested in this system. A 50 kDa protein was only synthesized in pWKS1030 (data not shown) and pFN23A carrying a 2.3 kb *SphI* fragment, suggesting that the full-length *ibeA* gene most likely resides in this region. As shown in Fig.2A, the reaction mixtures with the recombinant plasmid pFN23A containing the full length *ibeA* gene synthesized a 50 kDa protein, whereas the reaction mix with the vector alone did not synthesize such proteins. Synthesis of a 50 kDa protein by the *ibeA* construct in the *in vitro* translation system suggests that the size of IbeA protein is 50 kDa. For further confirmation of the size of IbeA, pFN23A was used for *in vivo* protein expression [Sankar P, et al, J Bacteriol (1993); 175:5145-52]. This expression system minimizes toxic gene effects under timing control of T7 RNA polymerase. The same size (50 kDa) of protein products was shown on the SDS-PAGE gel (Fig.2B). This protein band was isolated and subjected to N-terminal sequencing. The N-terminal amino acid sequence of IbeA was indicated in Fig.1. The size and N-terminal sequence of the expressed protein *in vitro* and *in vivo* perfectly matched that of the ORF deduced from DNA sequence data (Fig.1).

Example 5

Blocking *Escherichia coli* K1 Invasion of BMEC with the purified IbeA: The *ibeA* ORF of the DNA fragments cloned in this work was expressed in *Escherichia coli* BL21 (DE3)/pET28a expression system to examine the function of the expression product. This construct expresses a fusion protein (His₆-IbeA) that is composed of a His₆ tag and the entire *ibeA* ORF (50 kDa). When this authentic His₆-IbeA fusion protein was expressed in *Escherichia coli* strain BL21 (DE3), a 53-kDa protein was present in the inclusion body (Fig.3A). The His₆-IbeA protein was purified by Ni-NTA affinity chromatography from the insoluble fraction (Fig.3A). Refolding resulted in a soluble IbeA migrating with a single band of the same size (Fig.3B). The function of this protein was tested by examining the purified recombinant IbeA protein for the ability to inhibit invasion

of BMEC by strain E44 . 0.125 to 1.5 µg of the purified IbeA was pre-incubated with BMEC for 1 h at room temperature. The bacteria were added to the confluent monolayer of BMEC for invasion assay. As shown in Fig.4, the IbeA protein effectively inhibited *Escherichia coli* K1 invasion of BMEC in a dose-dependent manner, whereas the control protein BSA showed no such inhibition. Neither IbeA nor BSA affected bacterial viability under the experimental conditions used. More importantly, the entire recombinant IbeA protein showed much higher efficiency (50 times) in blocking the invasion of BMEC by *Escherichia coli* K1 than the partial protein fragment [Huang SH, et al, Infect Immun (1995); 63:4470-5], indicating that functional activities are greater with the whole protein.

Example 6

Construction and complementation of the *ibeA*-deletion mutant ZD1. To address the question whether the non-invasive phenotype of the TnphoA mutant 10A-23 is due to a polar effect, we constructed a mutant, ZD1 carrying 95% deletion of the *ibeA* ORF. The deletion of *ibeA* was confirmed by PCR and DNA sequencing. The non-invasive phenotypes of 10A-23 and ZD1 were shown in Fig.5. Both pUC23A carrying the entire coding region of *ibeA* gene and pUC1030 with a larger *ibeA* locus were able to completely restore the invasion ability of the non-invasive mutant ZD1 (Fig.5B). pUC1030 was able to completely complement the TnphoA insertion mutant 10A-23, while pUC23A was capable of partially restoring the non-invasive phenotype of 10A-23 (Fig.5A), suggesting that additional downstream gene(s) may be affected by the TnphoA insertion. However, pUC1030 is not sufficient to complement non-pathogenic *Escherichia coli* K12 strain HB101 (data not shown). These data provided further evidence to show that *ibeA* gene is one of the major determinants contributing to *Escherichia coli* K1 invasion of BMEC.

Table 1. *Escherichia coli* K1(meningitis-associated) or K12 (non-pathogenic) Strains, and Plasmids Used in this study.

5	Strains	Characteristics	Reference
	RS218	O18:K1:H7 (causing 32% of <i>Escherichia coli</i> meningitis)	(1)
	E44	RS218, Rif ^R	(1)
10	10A-23	E44 <i>ibeA</i> ::Tnp _h oA	(2)
	ZD1	Δ <i>ibeA</i> derivative of E44 via allelic exchange	This study
	DH5α (λpir)	K 12 strain	(3)
	SM10 (λpir)	K 12 strain	(4)
15	Plasmid	Characteristics	Reference
	pFN476	Amp ^r , low copy, T7 promoter	(5)
	pFN23A	pFN476 carrying <i>ibeA</i> gene (2.3 kb)	This study
	pUC13	Amp ^r , lacZ	(6)
	pWKS30	Amp ^r , lacZ	(7)
	pWKS1030	pWKS30 carrying <i>ibeA</i> locus (18 kb)	This study
20	pUC1030	pUC13 carrying <i>ibeA</i> locus (18 kb)	This study
	pUC23A	pUC13 carrying <i>ibeA</i> gene (2.3 kb)	This study
	pET28a(+)	Kan ^r , F1 origin, His•Tag	Novagen
	pCR17A	pCRII carrying <i>ibeA</i> gene (1.7 kb)	This study
	pET17A	pET28a(+) carrying <i>ibeA</i> gene (1.7 kb)	This study
25	pCVD442	Amp ^r , oriR6K, <i>sacB</i> , mobRP4	(3)
	pVZD	pCVD442 carrying DNA with Δ <i>ibeA</i>	This study

References for Table 1:

- 30 (1) Hoffman JA, et al, Infect Immun (2000); 68:5062-7; Huang SH, et al, Infect Immun (1995); 63:4470-5; Huang SH, et al, Infect Immun (1999); 67:2103-9;
 (2) Huang SH, et al, Infect Immun (1995); 63:4470-5;
 (3) Donnenberg MS, et al, Infect Immun. (1991); 59:4310-7;
 (4) Donnenberg MS, et al, Infect Immun (1990); 58:1565-71; Donnenberg MS, et al, Infect Immun. (1991); 59:4310-7;

- (5) Sankar P, et al, J Bacteriol (1993); 175:5145-52;
 (6) Wang Y, et al, Infect Immun (1999); 67:4751-6;
 (7) Wang RF, et al, Gene (1991); 100:195-9

5

Table 2. Oligonucleotides used for cloning, sequencing and making the deletion mutant of *ibeA* gene.

	Strains	Characteristics	Reference
10	10A5-4a	+	5'-TTGATCCCCGTACGCTTTC-3'
	10ZM6	-	5'-ACGCGTGGGTTCCAGATAAAATTCC-3'
	10D5M	+	5'-AGACGCGTCAGGAACGCTTACAGC-3'
	10D3	-	5'-CAAACCATCAGAACCGG-3'
	10A5-3S	+	5'-CTTGTACTCGGGTTAGAG-3'
15	10B3-4a	-	5'-ATAACACCGATGCCAAC-3'
	10A5-3Sa	+	5'-AGTCGAC TTGTACTCGGGTTAGAG-3'
	10A3-1S	+	5'-GTCGACATATGTTTAGCCCTTATC-3'
	10A3-4a	-	5'-GCAGTGTACCTGCATAG-3'
	10A3-2	+	5'-TGAACGTTGTCAGCATC-3'
20	10A3-3	+	5'-CCCTAATGCCAACAATC-3'

DISCUSSION

Current evidence suggests that microbial penetration across the BBB and entry
 25 into the CNS are a result of the specific interaction of several bacterial determinants with
 BMEC, which is a major component of the BBB [Huang, SH, et al, *Microbes and
 Infection* (2000); 2:1237-44. Kim KS, et al, *Subcell Biochem* (2000); 33:47-59]. Our
 investigations of this issue with *Escherichia coli* have shown that successful traversal of
Escherichia coli across the blood-brain barrier requires two independent steps of
 30 bacterium-BMEC interactions, i.e., *Escherichia coli* binding to BMEC and invasion of
 BMEC. We have previously shown that S fimbriae contribute to *Escherichia coli* binding

to BMEC [Stins MF, et al, *Am J Pathol.* (1994); 145:1228-36]; however, binding to BMEC via S fimbriae was not accompanied by invasion of BMEC. We have subsequently identified several *Escherichia coli* K1 determinants contributing to invasion of BMEC, i.e., IbeA, IbeB, YijP, AslA, and OmpA [Hoffman JA, et al, *Infect Immun* 5 (2000; 68:5062-7. Huang SH, et al, *Infect Immun* (1995); 63:4470-5. Huang SH, et al, *Infect Immun* (1999); 67:2103-9. Wang Y, et al, *Infect Immun* (1999); 67:4751-6. Prasadarao NV, et al, *Infect Immun* (1996); 64:146-51]. IbeB, yijP, aslA, and ompA have homologues present in the *Escherichia coli* K12 genome, while the *ibeA* gene was found to be a specific virulence factor present in CSF isolates of *Escherichia coli* K1 [Huang 10 SH, et al, *Infect Immun* (1995); 63:4470-5. Bingen E, et al, *J Infect Dis* (1998); 177:642-50. Bonacorsi SP, et al, *Infect Immun* (2000); 68:2096-101].

We have previously shown that the TnpHoA mutant of *ibeA*(10A-23) was significantly less invasive in BMEC monolayers *in vitro* as well as in the newborn rat model of hematogenous meningitis than the parent strain. A small recombinant protein 15 fragment (8.2 kDa) encoded by the partial *ibeA* gene was able to block *Escherichia coli* K1 invasion of human BMEC [Huang SH, et al, *Infect Immun* (1995); 63:4470-5]. In further characterization of the *ibeA* gene, we have several lines of evidence showing that the product encoded by the full-length *ibeA* gene was a 50-kDa protein. The first evidence that IbeA is synthesized as a 50 kDa protein came from transcription/translation 20 experiments performed in an *Escherichia coli* T7 S30 extract assay system. This data was further supported by the detection of the same size of a gene product by expression and purification of the IbeA protein from *in vivo* protein expression systems. The first 20 residues of the N-terminal sequence of the purified protein completely matched that of the deduced ORF from the DNA sequence data. These results indicate that the size of the 25 IbeA protein is 50 kDa. The deduced protein sequence suggests that IbeA is a potential membrane protein with three transmembrane domains (Fig. 1). However, no significant homology was found with known genes including any other recognized invasion proteins, suggesting that IbeA is unique to the known microbial virulence proteins.

As described previously, *ibeA* gene was initially identified by TnphoA mutagenesis [Huang SH, et al, *Infect Immun* (1995); 63:4470-5]. In order to exclude the possibility that a non-invasive property of 10A-23 is related to a polar effect of TnphoA on the other genes involved in invasion, an isogenic deletion mutant of *ibeA*, ZD1, was made and tested for its invasion phenotype in BMEC. Like the TnphoA mutant 10A-23, the deletion mutant ZD1 was less invasive in BMEC. The *ibeA* gene encoding the 50 kDa IbeA protein was able to partially and completely restore the non-invasive phenotype of 10A-23 and ZD-1 mutants, respectively. However, pUC1030 carrying a 18 kb *ibeA* fragment was capable of completely restoring the invasion ability of both 10A-23 and ZD1 to the level of the parent strain E44, suggesting that additional downstream gene (s) might be affected by the TnphoA insertion in the *ibeA* gene. Furthermore, *Escherichia coli* K12 strain HB101 was not complemented by pUC1030 (data not shown), implying that additional determinants may contribute to internalization and intracellular survival of *Escherichia coli* in BMEC. It remains to be determined how multiple different invasive determinants of *Escherichia coli* K1 contribute to crossing of the blood-brain barrier.

We have recently identified a 55 KDa membrane protein from BMEC, which binds to a partial fragment of IbeA (8.2 kDa). A soluble form of this IbeA receptor (IbeAR) and a polyclonal antibody against the IbeAR block *Escherichia coli* K1 invasion of BMEC [Prasadarao NV, et al, *Infect Immun* (1999); 67:1131-8]. We have previously shown that the partial IbeA fragment was able to inhibit *Escherichia coli* K1 invasion of BMEC but high dose (50 µg) was required for approximately 80% inhibition [Huang SH, et al, *Infect Immun* (1995); 63:4470-5]. However, as shown in this report, we demonstrated that the entire recombinant 50 kDa IbeA protein was much more efficient in blocking *Escherichia coli* K1 invasion of BMEC and only 1 µg dose achieved a similar degree of inhibition. Taken together, these findings suggest that the previously reported 8.2 kDa fragment of IbeA represents a part of the binding domain interacting with IbeAR, but other structures of IbeA are required for optimal interaction with BMEC.

In conclusion, our data further support that the *ibeA* is a major determinant for *Escherichia coli* K1 invasion of BMEC, an essential step in the pathogenesis of *Escherichia coli* meningitis. Studies are in progress to define the mechanisms by which *ibeA* locus contributes to *Escherichia coli* K1 invasion of the blood-brain barrier.

5 Example 7-12

In order to identify *E. coli* structures that contribute to the invasion of the blood-brain barrier (BBB), we have used transposon TnphoA mutagenesis to generate a collection of noninvasive mutants. Four noninvasive mutants, 10A-23, 7A-33, 23A-20, and 27A-6 with a single TnphoA insertion and without any changes in other phenotypic and genotypic characteristics, were found to be significantly less invasive of BMEC monolayer *in vitro* and of the central nervous system in the newborn rat model of hematogenous *E. coli* meningitis (Huang et al., (2000). Microbes and Infection. 2:1137-44; Huang, and Jong (2001) Cellular Microbiology; Huang et al., (2001) J. Infect. Dis. 183:1071-8; Hoffman, J.A. et al (2000) Infect. Immun. 68:5062-5067). Accordingly, four *E. coli* genetic determinants *ibeA*, *ibeB*, *yijP*, and *aslA* contributing to *E. coli* invasion of the BBB have been identified. *IbeB*, *yijP* and *aslA* have homologues present in the non-pathogenic *E. coli* K-12 genome. However, the *ibeA* gene encoding a 50-kDa protein has been found to be unique in cerebrospinal fluid (CSF) isolates of *E. coli* K1 (e.g., C5 and RS218), while laboratory strains of *E. coli* K-12 (e.g., DH5 and HB101), as well as noninvasive *E. coli* (e.g., E412), lack *ibeA* (Huang. et al., (1995) Infect. Immun. 63:4470-4475; Huang et al., (2001) J. Infect. Dis. 183:1071-8; Johnson, JR et al., (2001) J Infect Dis. 183:425-34). The *ibeA* locus was able to completely complement the TnphoA insertion and isogenic deletion mutants 10A-23 and ZD1 (Huang et al., (2001) J. Infect. Dis. 183:1071-8.

25 We report here a 20.3-kb locus including the *ibeA* gene that is found to be unique in *E. coli* K-1 strains. It is situated between *yjiD* and *yjiE*, adjacent to the *fim* operon, and absent in non-pathogenic *E. coli* strains. It has regions different in G+C content from the

rest of the genome, indicating that this gene cluster is a genetic island of meningitic *E. coli* containing *ibeA* (*gimA*). Additional 14 novel open reading frames (ORFs) have been identified.

Materials and Methods for Examples 7-12

5 **Bacterial strains, plasmids and media.** *E. coli* strain RS218 (O18:K1:H7) is a clinical isolate from the CSF of a neonate with meningitis (Huang, S.H. et al., (1995) *Infect. Immun.* 63:4470-4475). E44 is a spontaneous rifampin-resistant mutant of RS218, which has been characterized (Huang, S.H. et al., (1995) *Infect. Immun.* 63:4470-4475; Huang, S.H. et al., (1999) *Infect. Immun.* 67:2103-2109; Huang, SH et al., (2001) 10 *J. Infect. Dis.* 183:1071-8.). DH5 α was used as the host strain in subcloning and preparation of plasmids for DNA sequence determination. Strains containing plasmids were grown at 37⁰C in L broth with ampicillin (100 μ g/ml), kanamycin (50 μ g/ml) for positive selection of plasmids. Bacteria were cultured in L broth and stored in L broth with 20% glycerol at -70⁰C.

15 **Chemicals and enzymes:** Restriction endonucleases, T4 DNA ligase, and other enzymes were purchased from New England Biolabs (Beverly, Mass.) unless otherwise noted. Chemicals were purchased from Sigma (St. Louis, MO). All isotopes were obtained from New England Nuclear Corp. (Boston, Mass.). Reagents for preparation of DNA sequencing gels were ultra pure quality from National Diagnostics (Atlanta, 20 Georgia). The reagents for DNA sequencing reaction with Sequenase and other chemicals were purchased from United States Biochemical Corp. (Cleveland, Ohio). DNA sequencing kits with dye terminators were obtained from PE Applied Biosystem (Great Britain).

25 **Extraction and manipulation of plasmids and subcloning.** All genetic manipulations were performed by standard methods (Sambrook, J., et al., (1989). Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring). Plasmid All isotopes

were obtained from New England Nuclear Corp. (Boston, Mass.). DNA was extracted by using a Plasmid Mini kit (Qiagen Inc., Chatsworth, Calif.). Purification of DNA fragments and extraction from agarose gel slices were performed with GeneCleanTM (Bio 101, La Jolla, Calif.). Competent cells of *E. coli* were made in 10% glycerol and transformed with electroporation (Huang, S.H. et al., (1999) Infect. Immun. 67:2103-2109; Huang SH et al., (2001) J. Infect. Dis. 183:1071-8). Genomic DNA was partially digested with *SauA3 I* (New England Biolabs), which is compatible with BamHI. Partially digested genomic DNA (15 to 23 kb) was partially filled-in with dGTP and dATP. This DNA was ligated into LambdaGEMTM-12 arms with XhoI Half-site. Ligation and packaging of recombinant lambda phage were performed according to the manufacturers instructions (Promega). The *E. coli* genomic library was screened by DNA hybridization (Huang et al, 1999, 2001) to identify phage clones that contained *ibeA*. A 0.58-kb *ibeA* DNA fragment in pCIB10B (Huang et al 1995) was released with *EcoRI* and purified by preparative agarose electrophoresis and GeneClean (Bio101), labeled with [α^{32} P]dCTP by using an oligolabeling kit (Pharmacia), and used as a probe in the screening ($>1 \times 10^8$ cpm/ μ g). The phage plaques were replicated onto Nylon filters, UV-linked and hybridized as described previously (Huang et al 1999, 2001). Plaques hybridizing to the probe were identified by autoradiography and then purified.

Screening of *E. coli* RS218 genomic library by DNA hybridization. A genomic library of *E. coli* RS218 was constructed as previously described (Huang, SH et al., (2001) J. Infect. Dis. 183:1071-8). The *E. coli* genomic library was screened by DNA hybridization to isolate phage clones that contained the *ibeA* locus. A 0.58 kb *ibeA* DNA fragment was purified by preparative agarose electrophoresis and GeneCleanTM (Bio101), labeled with [32 P]dCTP by using an oligolabeling kit (Pharmocia), and used as a probe in the screening ($>1 \times 10^8$ cpm/ μ g). The phage plaques were replicated to Nylon filters, UV-linked and hybridized as described previously. Plaques hybridizing to the probe were identified by autoradiography, and then purified.

PCR. PCR was carried out by a standard method. The left junction region was amplified by YjD1 (5'-AAT GCT GTA CCA CGA CG-3') and 8T3a (5'-T CAT AGT CTA CGT CTC GCC GAC-3'), using 30 cycles of 94°C for 1 min, 58 °C for 1 min, 70 °C for 3 min.

5 **Preparation of templates for nucleotide sequencing.** Two overlapping genomic clones (10-8 and 10-30) and a PCR clone, prepared from genomic DNA from *E. coli* RS218 and found previously to carry a DNA sequence specific to *E. coli* K1 strain, were used for nucleotide sequencing through primer walking.

10 **DNA sequencing and analysis:** At first the partial DNA sequence of the *gimA* was determined by the dideoxy chain termination method of Sanger et al with Sequenase version 2.0 kit from U.S. Biochemicals Corp. (Cleveland, Ohio) and Taq DNA polymerase. DNA sequence analysis was performed manually with [³⁵S]dATP(1,000 to 1,500 Ci/mmol) obtained from Du Pont NEN Research Products (Boston, Mass.). Subsequently we switched to the automatic DNA sequencing approach with
15 fluorescence labeled nucleotides (373A ABI automated sequencer) because of higher speed, lower cost and avoiding electrophoretic compression artifacts derived from the high G + C content. The entire *ibeA* gene cluster was sequenced by primer-walking. Both strands of the DNA were sequenced to ensure the accuracy.

BIOINFORMATICS APPROACHES

20 The sequence data was analyzed with the DNA analysis programs developed by Scientific & Education Software (Durham, NC). DNA and deduced protein sequences were used to search the DNA and protein data bases at the National Center for Biotechnology Information (National Library of medicine, Washington, D.C.) by using the BLAST algorithm. ClustalW (Thompson JD et al (1994) Nucleic Acids Res.
25 22:4673-4680) and Boxshade (Hofmann K et al., (1999) Nucleic Acids Res. 27:215-9) were used for multiple alignment of protein sequences. Annotation of genes from the

genetic island gimA makes use two conceptually different approaches. First, sequences are compared to existing sequences with known functions at the protein level to identify homologous regions and then to predict the functionality of the novel genes. Second, a uniform Hidden Markov Model for annotation of prokaryotic genes is used to predict the unknown genes in gimA (Shmatkov AM et al., (1999) Bioinformatics 15:874-86). Since tightly packed prokaryotic genes frequently overlap with each other, it is easy by targeting gene starts and overlapping genes to makes detection of translation initiation sites and, therefore, exact predictions of prokaryotic genes. A combination of the comparative analyses and the 'frame-by-frame' algorithm leads to annotation of a given novel gene. Phylogenetic analysis was performed by using BioNavigator (<http://www.bionavigator.com>).

Example 7

Cloning and subcloning of the *ibeA* gene cluster of *E. coli* RS218.

E. coli K1 strain RS218 was used as the source of DNA for cloning experiments. This CSF isolate was capable of invading human and bovine brain microvascular endothelial cells (BMEC) and inducing meningitis subsequent to bacteremia in newborn rats. To clone the *ibeA* determinants from RS218, a genomic library was constructed in LambdaGEM-12. By using half sites of XhoI in the vector and SauA3I in the genomic inserts for library construction, self-ligation of vector and genomic sequences was eliminated since only recombinant phages containing a single insert of the appropriate size (15-23 Kb) were capable of being packaged. Approximately 5×10^5 recombinant phages were screened by using an *ibeA* fragment (0.58 Kb) as a probe. Three overlapping phage clones for *ibeA* were identified. The recombinant phage DNAs were purified and digested with NotI. The size of the two overlapping inserts was 17.3 (A10-8) and 18.0 (A10-30) Kb, respectively. These inserts containing *ibeA* gene cluster were subcloned into NotI site of pBluescript KS (pKS108 & pKS1030).

Example 8

Identification of the junction regions.

The right junction (where “right” is defined as the sequences associated with higher numbers on the linkage map of *E. coli* K12) was identified by T3 primer on the A10-30 clone. A stretch of 530 bp sequence was determined. A search of GenBank revealed that this region is identical to the C-terminal coding sequences of *yjiD* (12 bp) and *yjiE* (518 bp) in *E. coli* K12. The *gimA* insertion site is located inside *yjiD* ORF, 9-bp upstream of *yjiD* stop codon, and outside *yjiE* ORF, 4 bp upstream from *yjiE* stop codon. 18-kb sequences were obtained from A10-8 and A10-30 clones. According to the physical mapping (Bloch et al. 1996), *gimA* is about 20 kb in size. In order to identify this sequence gap, a 5'-primer (10YjD1) and a 3'-primer (8T3a) were picked up from 62 bp upstream of *yjiD* stop codon and the 5' region of the known *gimA* sequence, respectively. To isolate and verify the left junction of *gimA*, these two primers were used for PCR to amplify this region directly from E44 genomic DNA. A 2.4 kb DNA was isolated and subcloned into pGEM-T easy vector (E44L7). Sequence analysis of this PCR clone defined the left junction and revealed that a 2.3 kb sequence is unique to *E. coli* K1 and has a 30 bp overlapping region with the known *gimA* sequence. Therefore, *gimA* is 20.3 kb in size.

Example 9

Nucleotide sequences of *gimA*.

The DNA sequences of the *ibeA* gene cluster were determined by a primer walking approach with primers complementary to both strands of *gimA* genes in A10-8, A10-30 and E44L7. Analysis of the DNA sequence data indicated that *gimA* is located in 98 min. Two phage clones (A10-8 and A10-30) shared an overlap region in which they have the identical *ibeA* and the downstream region (Figure 6). The results of open reading

frame (ORF) analysis and the features of the *gimA* were summarized in Figures 7 and 8 and Table 4.

Example 10

Novel genes inside the *gimA*.

5 To determine ORFs present within the *gimA*, the entire gene cluster was subjected to nucleotide sequencing. In this region, which included an apparent 20.3-kb *gimA*, we have identified 15 ORFs including the known gene *ibeA* (Figure 9). Among the sequenced ORFs are genes that appear to be involved in carbohydrate metabolism, transport systems, protein binding and transcriptional regulation (Table 4).

10 Example 11

Insertion sequences and transposons. No known insertion sequences have been identified from *gimA* and its junctions. However, a search of GenBank indicated that a 57 bp region (5358-5414) shares significant homology (85%) with transposon Tn5542 in *Pseudomonas putida*.

15 Example 12

Other features of the *gimA*. The *gimA* of strain RS218 displays two other features common to such blocks of virulence genes. First, the GC content of the sequences, not including those of K-12 origin, is 46.2%. This value is significantly different from a value of 50.8% for the *E. coli* genome. Also, the *gimA* sequence is
20 unique to *E. coli* K1 strain and does not share significant homology with sequences of K-12 origin.

DISCUSSION

We have characterized a 20.3-kb region of DNA from *E. coli* RS218, a CSF isolate from a newborn baby with *E. coli* meningitis, by isolation of overlapping phage and PCR clones, restriction endonuclease mapping, subcloning, and DNA sequencing (Figure 7). In this region, we have identified what can be defined as a genetic island that includes 15 ORFs (Table 4 and Figure 9). Genetic or pathogenicity islands typically have a GC content lower than that of neighboring DNA, and have gene clusters positioned near each other which contribute to a single virulence property. The *gimA* of RS218 is 20.3 kb in size (Figure 8), has a GC content of 46.2% (compared to 50.8% in K-12 genomic DNA).

Nucleotide sequences of the 20.3-kb *gimA* of RS218 reveal 14 newly described genes. The *ibeA* gene has been found essentially in *E. coli* meningitis and significantly more frequently detected in strains that are positive for the *sfafoc* operon than in strains that are negative for the *sfafoc* locus. Therefore, we postulate that these newly described genes along with *ibeA* may represent a genetic island that contributes to the pathogenesis of neonatal meningitis caused by *E. coli*. Insertion and deletion in *ibeA* gene led to a non-invasive phenotype of RS218 *in vitro* and *in vivo*, suggesting that this virulence determinant contributes to *E. coli* invasion of the blood-brain barrier (Huang, S.H. et al., (1995) Infect. Immun. 63:4470-4475; Huang, SH et al., (2001) J. Infect. Dis. 183:1071-8). As we continue our studies on the genetic island of RS218, we will select more mutants with phenotypes that may relate to virulence such as metabolite uptake and transcriptional regulation. We will undertake allelic exchange mutagenesis of specific genes and test these isogenic mutants by *in vitro* BMEC invasion assays and, *in vivo*, by using the neonatal rat model of meningitis. By creating such mutants, we hope to identify additional genes of *gimA* contributing to the virulence phenotype of meningitic *E. coli*. It is likely, however, that meningitic *E. coli* strain RS218 contains additional genetic islands. For two uropathogenic strains that have been studied closely, 536 and J96, each strain was found to contain two separate pathogenicity islands. For strain

536, pathogenicity islands of 190 and 70 kb are inserted at 97 min (within *leuX*) and 82 min (within *selC*), respectively. For strain J96, PAIs of 110 and >170 kb are inserted at 94 min (within *pheR*) and 64 min (within *pheV*), respectively. Mapping of non-invasive TnphoA mutants revealed that at least two putative genetic islands, including
5 gimA and a cluster of 12 TnphoA insertions located at a 120 kb RS218-specific chromosomal segment, are present in RS218 genome (Bloch, C.A. et al., (1996) FEMS Microbiol. Lett.,144: 171-176).Bloch et al. 1996). Very recently, Bloch's lab (Rode CK, et al., (1999) Infect Immun. 67:230-236) and Bingen's group (Bonacorsi SP et al., (2000) Infect Immun. 68:2096-2101) detected several new additions in RS218 genome
10 by using comparative macrorestriction mapping and representational difference analysis, respectively. That RS218 may have additional genetic islands of unknown size raises the possibility that a significant number of other virulence genes, not detected on the gimA described here, may also contribute to the virulence of this strain.

Table 3. Oligonucleotides used for cloning and sequencing.

	Primer	Strand	Sequence
5	8T3a	-	5'-T CAT AGT CTA CGT CTC GCC CAG-3'
	8T3b	-	5'-TCA ACG AAC TGG CAA TGC TG-3'
	8T3c	-	5'-CTA TTA CCC CGC AAA ACG TC-3'
	8T31	+	5'-GCA ACC ATA ATT TAT CCC GCG-3'
	8T32	+	5'-CGG CCA TAT CTA ATG ATG TAC-3'
10	8T33	+	5'-GCT ATC TTT TAC CGC TAC ATC-3'
	8T34	+	5'-GGA TGA TGT TTT TTA CAG CGC G-3'
	8T35	+	5'-ACA CTG GCG GCA CTG GCT ATT G-3'
	8T36	+	5'-AGC GAC TAA TGC TGA ACT TGG-3'
	8T32a	-	5'-TAT TTA TGT GCG CCG CAC AG-3'
15	8T33a	-	5'-GAT GTA GCG GTA AAA GAT AGC-3'
	8T3ba	+	5'-AGG ATG GCG TGA GTT GCT GC-3'
	8T3bb	+	5'-CCA GCA TTG CCA GTT CGT TG-3'
	10A5-2	-	5'-GGTATATTACGAGCGGG-3'
	10A5-3	-	5'-ATCTTCAGCTGCTTTAGTTAG-3'
20	10A5-4	-	5'-CTTCACGACGTTTGCGC-3'
	10A5-5	-	5'-AATTTTCCCACACCTTCT-3'
	10A5-6	-	5'-CGGCGGAAATACGAATC-3'
	10A5-7	-	5'-CAT GAC CTC AGC ATC AC-3'
	10A5-8	-	5'-GGC GTG TGT ATT GGC ACA TC-3'
25	10A5-9	-	5'-TCT GAT GCT TGA AAA GCG CC-3'
	10A5-10	-	5'-TGA CGA ATT TCA CGT ACC TG-3'
	10A5-11	-	5'-TAA CAA CAC CAG ACA AGC CC-3'
	10A5-4a	+	5'-TTGATCCCCGTACGCTTTC-3'
	10A5-5a	+	5'-GGGCAATTAAATCCATCTCTCC-3'
30	10A5-8a	+	5'-CAT GAC GGG CCA GAA TAT G-3'
	10A5-9a	+	5'-ACA GGC ATT AAT CCA GTG GC-3'
	10A5-11a	+	5'-ACA CTC CTG CGC GAC TTC-3'
	10A3-4	+	5'-AATTTTCAGCGGCGTTTTCC-3'
	10A3-5	+	5'-AGTGATACCACCAACC-3'
35	10A3-7	+	5'-TGGCTGTATCAAGGTTTC-3'
	30T71	+	5'-GAC TAT CTA ATT TCC CTT CAC CG-3'
	30T72	+	5'-CCT TGA ACT TGT GCC AGT TC-3'
	30T73	+	5'-TAT TCA ACA GGC GGG CAT TC-3'
	30T73a	-	5'-AAA GGA ACA TTC GAACCC GG-3'
40	30T7a	-	5'-AAT TTA CCG ACC GCG CTG AGTC-3'
	30T7b	-	5'-CGG TAC TTA AAC TCA TCG CTA C-3'
	30T7c	-	5'-CAT AAC GTG AGA AGG CCA GC-3'
	30T7d	-	5'-GAC GCA CGG TGC AAT TTT GC-3'
	30T7e	-	5'-CAG TTT TTG CCC CAA TCC GC-3'
45	30T7f	-	5'-CGC AAT CCG CAT TGT TTT GAG-3'
	30T7Ba	+	5'-GAA TCG TCG CCA TCA CAC TC-3'
	30T7BB	-	5'-AGC CGA AAT TAG CCA GTA CC-3'

30T7CB	+	5'-AAA AAA GGT GTG GCA TGG GC-3'
30T7Da	+	5'-TAT TTC CGC AGG CGT AGT TGC-3'
L7SP1	+	5'-CCA TCG GCA GCA TAA TTT GC-3'

Table 4. Gene annotation of the *ibeA* gene cluster (*gimA*)

Operon	ORF	Size (aa)	Start Position	Start codon	Accession Number	Homologous to	Functions
GimA1 (PTS)	<i>PptE</i>	536	1694C	GTG	AF289032	BACST PEP-PPT (213/542=39%); <i>E. coli</i> PT1 (203/540=37%)	phosphoenolpyruvate-protein phosphoryltransferase (PEP-PPT)(enzyme I)
	<i>PmpT</i>	214	2466C	ATG	AF289032	<i>E. coli</i> hypothetical protein YCGC (98/212=46%); XANCP PTF1/MTP (46/182=25%)	multiphosphoryl transfer protein (MTP)
	<i>PgdK</i>	210	3102C	ATG	AF289032	CITFR glycerone kinase (53/199=26%)	Putative glycerone kinase
	<i>PdaK</i>	356	4185C	ATG	AF289032	<i>E. coli</i> hypothetical protein YCGT (211/355=59%); PICAN DHA kinase (136/364=37%)	Putative DHA kinase
GimA2 (Glycerol metabolism)	<i>CgrD</i>	360	4504	ATG	AF289032	Glycerol dehydrogenase CITFR (225/359=62%; <i>E. coli</i> (204/359=56%)	Glycerol dehydrogenase
	<i>CgxT</i>	422	5653	ATG	AF289032	<i>E. coli</i> hypothetical protein YIHN (137/414=33%);	Putative transporter
	<i>CdlD</i>	454	6939	ATG	AF289032	<i>E. coli</i> dihydrolipoamide dehydrogenase (25/44=56%)	Putative dihydrolipoamide dehydrogenase?
	<i>CniT</i>	443	8604	ATG	AF289032	<i>E. coli</i> CAIT (130/404=32%) BACSU glycine betaine transporter (123/366=34%)	Putative carnitine transporter (CAIT)
GimA3 (enolase super-family)	<i>GcxK</i>	380	11344C	ATG	AF289032	<i>E. coli</i> hypothetical protein YBBZ (171/372=45%)	Glycerate kinase (glxK)
	<i>GcxR</i>	295	12306C	ATG	AF289032	<i>E. coli</i> hypothetical protein YBBQ (166/290=57%); PSEAE HIBADH (85/249=34%)	Tartronate semialdehyde reductase (glxR)
	<i>GclA</i>	579	14858C	ATG	AF289032	<i>E. coli</i> glyoxylate carboligase (393/578=67%)	Glyoxylate carboligase (gcl)
	<i>Ghyl</i>	260	13106C	ATG	AF289032	<i>E. coli</i> glyoxylate induced protein & hydroxypyruvate isomerase (137/254=53%)	Hydroxypyruvate isomerase (hyi/gip)

Operon	ORF	Size (aa)	Start Position	Start codon	Accession Number	Homologous to	Functions
GimA4 (regulation of gimA)	<i>lbgR</i>	624	15237	ATG	AF289032	DHAR Glycerol metabol. Operon (GMO) regulator (186/590=31%)	Putative GMO regulatory protein
	<i>lbeA</i>	456	17476	ATG	AF289032	DROME AMAN II (27/97=27%); PLAVS erythrocyte binding protein (18/64=28%)	Invasion protein contributing to <i>E. coli</i> invasion of the blood-brain barrier in vitro and in vivo; binding to IbeA receptor on brain endothelial cells.
	<i>lbgT</i>	467	18861	ATG	AF289032	HAEIN Na(+)/H(+) antiporter (148/435=34%)	Putative Na(+)/H(+) transporter

C = complementary strand

Example 13 Induction of Apoptosis by IbeA

Several studies suggested that hippocampal apoptosis was a characteristic feature of bacterial meningitis in human disease and in several animal models of meningitis (Nau R et al., J Neuropathol Exp Neurol. 1999, 58(3):265-74; Loeffler JM et al., J Infect Dis. 2001, 183(2):247-252.; Bottcher T et al., J Infect Dis. 2000, 181(6):2095-8; Braun JS et al., Nat Med. 1999, 5(3):298-302; Leib SL et al., J Clin Invest. 1996, 98(11):2632-9). As shown in our previous studies, IbeA-dependent *E. coli* K1 invasion was observed in both intestinal epithelial and brain endothelial cells. Since apoptosis is a key feature of bacterial meningitis, we proposed that apoptosis might be associated with E44 invasion.

In order to determine whether apoptosis plays a role in pathogenesis of *E. coli* meningitis, we tested apoptotic activity of IbeA and IbeB proteins. Human EC were grown on collagen coated eight-well chamber slide. At confluence, human EC were treated with 5 µg of protein [purified *E. coli* protein IbeA, IbeB, BPI or bovine serum albumin (IbeB and BSA are used as control)] and incubated for up to 6 hrs at 37°C. Subsequently, human EC were washed three times with experimental medium, fixed with 1% paraformaldehyde in PBS at 4°C (Stins, M.F.et al., J. Neurovirol. In press). Apoptotic cells were detected by ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Briefly, the 3'-OH DNA ends that were generated by DNA fragmentation become substrates for terminal deoxynucleotidyl transferase (TdT) and then digoxigenin nucleotides were catalytically added to the apoptotically produced DNA ends. These nucleotides were detected by antidigoxigenin antibody carrying a conjugated peroxidase. Diaminobenzidine was then reacted with peroxidase to produce insoluble brownish-colored products in apoptotic bodies where DNA fragmentations were present. The slides were then viewed and photographed in the microscope (Stins, M.F.et al., J. Neurovirol. In press.). As shown in Fig.13, IbeA was capable of inducing apoptosis in human EC comparing to the treatments with BPI and BSA plus IbeB, and BPI was able to block IbeA-induced apoptosis. Under the same conditions another purified invasion protein IbeB (Huang SH et al.,1999) was unable to induce apoptosis in human EC. Taken together, our data demonstrate that IbeA is a major

apoptotic factor in E44 contributing to induction of apoptosis in human EC, which is inhibited by the anti-endotoxin protein BPI (Arditi, M. et al., Infect. Immun. 1994, 62:3930-6.). In many brain diseases including bacterial meningitis, neuronal damage or death is due to apoptosis. Blocking IbeA-induced apoptosis by anti-apoptotic agents such
5 as BPI will be a novel approach to prevention and treatment of neonatal *E. coli* meningitis.